superior to radio-isotopic tracer methods in terms of dose size and analysis efficiency [136]. Guo et al. reported that GC–C–IRMS provides 15-fold lower detection limits for [$^{13}C_2$-C3,C4]cholesterol than organic GC–MS [137]. Using [U-^{13}C]α-linolenic acid, Sheaff et al. were able demonstrate that conversion of α-linolenate into docosahexaenoate was not depressed by high dietary levels of linoleic acid [117]. An interconversion of saturated dietary fatty acids (e.g., 18:0) into unsaturated fatty acids (e.g., 18:1) in plasma of about 14% was reported by Rhee et al. [138]. Menand et al. applied this technique to measure carbon incorporation into plasma glutamine [139]. Practical aspects of this technique have been investigated by Dube et al. [140,141].

4. Compound-specific isotope analysis of ^{15}N isotopic abundance

High precision CSIA of ^{15}N isotopic abundance faces three major challenges. First, the relatively low concentration of nitrogen in organic compounds (for instance, amino acids contain two to 11 times more carbon than nitrogen). Secondly, $^{15}N/^{14}N$ isotope ratios are measured on N_2, which means the compound conversion step requires a reduction process and, in the case of amino acids, the formation of one mol equivalent of N_2 produces 4–22 molequivalents of CO_2 (if we regard the underivatised case). Lastly, even small amounts of atmospheric gas leaks into the instrument result in a high N_2 background.

Due to the wide spectrum of applications that would benefit from a system capable of analysing ^{15}N isotopic enrichment down to natural abundance level, especially because there is no radioactive alternative, these difficulties did not delay instrument development for too long. The desire to study for example nitrogen fixation in plants and micro-organisms and nitrogen metabolism in humans, as well as the possibility to quantify gene expression by measuring mRNA turnover, prompted two groups to extend the scope of GC–C–IRMS. In 1994, Preston and Slater presented a system with a conversion interface comprising combustion furnace, liquid N_2 cold trap, to trap CO_2 and water, and a PLOT column to resolve N_2 from any CO formed by poor combustion [142]. If CO were permitted to enter the ion source simultaneously with N_2, this would result in a serious isobaric interference at m/z 28. For δ^{15}N values of *t*BDMS derivatives of amino acids, they reported a precision of S.D. (δ^{15}N)=5‰ at natural abundance level.

In the same year, Merritt and Hayes presented a similar system but for the addition of a reduction furnace, loaded with Cu wires and maintained at a temperature of 600°C, to reduce N-oxides to N_2 and to scavenge O_2 emanating from the combustion furnace [143]. Their system also included a cryogenic trap to remove CO_2 and water, and it produced a precision of S.D. (δ^{15}N)=0.2‰.

This marked difference in precision was attributed to different performances of the IRMS systems. Ongoing investigations in our laboratory seem to indicate that this difference might be caused by the simultaneous presence of NO and N_2 in the ion source. Placing a PoraPLOT Q capillary column of 0.32 mm internal diameter, maintained at 30°C between the water trap and the ion source, separates N_2 from NO (Fig. 5). Comparing precision for δ^{15}N analysis of identical aliquots from the same sample with and without the PLOT column in place, we found a drop in precision from S.D. (δ^{15}N)=0.15‰ to S.D. (δ^{15}N)=2‰, respectively. This phenomenon cannot be explained by isobaric interference of NO (m/z 30) with m/z 28 ($^{14}N_2$) or m/z 29 ($^{15}N^{14}N$) but might be caused by reaction of NO with N_2 in the ion source.

To avoid potential isotopic fractionation due to various degrees of nitrogen silylation when TMS derivatives were formed, Hoffmann et al. prepared *t*BDMS derivatives of amino acids from wheat protein hydrolysate [144]. The same strategy was followed by Segschneider et al. when investigating ^{15}N uptake from ^{15}N-labelled NO_2 by measuring δ^{15}N values of soluble amino acids in sunflower leaves [145]. However, when preparing *t*BDMS derivatives, one has to bear in mind that in the case of, e.g., L-leucine, one adds 12 mol equivalent of carbon to 1 mol equivalent of amino acid, thus resulting in 36 mol equivalent of carbon for 1 mol equivalent of N_2, which might give rise to CO formation due to poor combustion. Metges et al. reported an alternative derivatisation protocol for amino acids yielding *N*-pivaloyl, *O*-isopropylates, and demonstrated their application by measuring NA

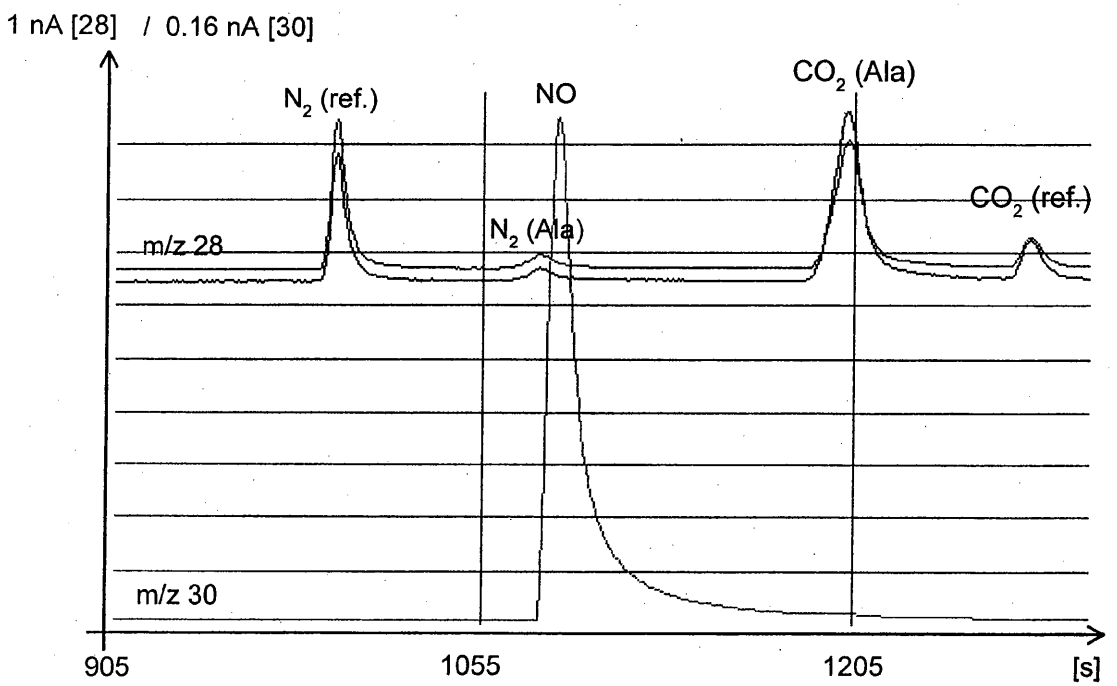

Fig. 5. The N_2^+ mass traces obtained for alanine (as *N*-acetyl, *O*-propyl derivative) having passed post-combustion through a PoraPLOT Q column, held at 30°C, shows the presence of NO next to N_2. The CO_2 peaks are caused by the formation of CO^+ from CO_2 in the ion source.

$\delta^{15}N$ values of amino acids from plasma albumin hydrolysate [146].

CSIA of ^{15}N isotopic abundance by GC–C–IRMS has been applied to measure the effect of ibuprofen on protein synthesis using ^{15}N-labelled glycine as tracer [147]. Williams et al. used ^{15}N-labelled urea to demonstrate that *Helicobacter pylori* uses urea as a nitrogen source for its synthesis of amino acids [148]. A multidisciplinary group comprising environmental, archaeological, geological and nutritional scientists showed that differences in $\delta^{15}N$ values from soil amino acids could be used to indicate differences in land use in Bronze Age, medieval and early modern soils [149]. One intriguing observation they made was the consistently low levels of ^{15}N abundance ($\delta^{15}N < 0.0‰$ vs air) in the amino acids threonine (Thr) and phenylalanine (Phe) from soils of unmanured cereal production sites, whereas $\delta^{15}N$ values of all the other amino acids were positive. We thought this was intriguing because a similar pattern was found by Metges and Petalec when monitoring $\delta^{15}N$ values of free plasma amino acids from fasting human subjects. With the exception of Thr and Phe, all other amino acids showed positive $\delta^{15}N$ values [150].

Mas et al. suggested that $\delta^{15}N$ values of MDMA (ecstasy) could be used for batch discrimination of ecstasy tablets [61], and Faulhaber et al. used $\delta^{15}N$ values of methyl-*N*-methylanthranilate as a biomarker in the authenticity control of mandarin oils [43]. Last, but not least, GC–IRMS was used for measuring $\delta^{15}N$ values of N_2 and N_2O, separated from the same sample [151,152].

5. Hyphenated techniques

In recent years, the research efforts of different groups working in the field of GC–IRMS have focused on extending the scope of on-line CSIA towards the measurement of organic $^{18}O/^{16}O$ and organic $^{2}H/^{1}H$ isotope ratios. Consequently, research

was undertaken with the aim of high-precision measurement of two different elemental isotope ratios such as $^{2}H/^{18}O$, $^{13}C/^{18}O$ and $^{13}C/^{15}N$, from the same compound source in one analytical run.

By placing a PoraPLOT Q capillary column between the combustion reactor and the IRMS (which enabled us to separate N_2 from CO_2 by 100 s baseline to baseline), $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratio from alanine, leucine and phenylalanine could be measured in one single analysis [153]. The excellent separation of N_2 from CO_2 provided ample time to switch IRMS ion source parameters from N_2- to CO_2-mode (Fig. 6).

In 1994, using a GC-based IRMS system, Brand et al. showed that CSIA of $^{18}O/^{16}O$ ratios was possible by converting oxygen-containing organic compounds on-line to CO by means of a pyrolytic reaction [154]. The on-line coupling of GC and IRMS via a pyrolysis interface (GC–Py–IRMS) was used for the simultaneous determination of $\delta^{13}C$ and $\delta^{18}O$ values for vanilla from different origins [155]. Farquhar et al. converted bulk plant matter into N_2 and CO by an automated on-line pyrolysis-based reaction using nickelized carbon at about 1100°C and separating N_2 from CO post-pyrolysis in a GC fitted with a 5-Å molecular sieve PLOT column [156].

Independently, Begley and Scrimgeour reported on high-precision $\delta^{2}H$ and $\delta^{18}O$ measurement for water and VOCs by using 20% nickelized carbon to generate both H_2 and CO at temperatures of between 1050 and 1100°C [157]. Precisions were S.D. ($\delta^{2}H$) = 2‰ and S.D. ($\delta^{18}O$) = 0.3‰ for samples ranging from urine, water and VOCs. Their pyrolysis system was based on earlier work that was aimed at simultaneous $\delta^{2}H$ and $\delta^{18}O$ determination from small water and urine samples (0.5 μl) [158]. Common to both studies was the use of a novel IRMS with the high dispersion necessary for separation of the $^{2}H^{1}H^{+}$ and $^{4}He^{+}$ ion beams. This novel high mass dispersion IRMS has been described in detail by Prosser and Scrimgeour [159]. Very recently, this high mass dispersion IRMS was coupled to a

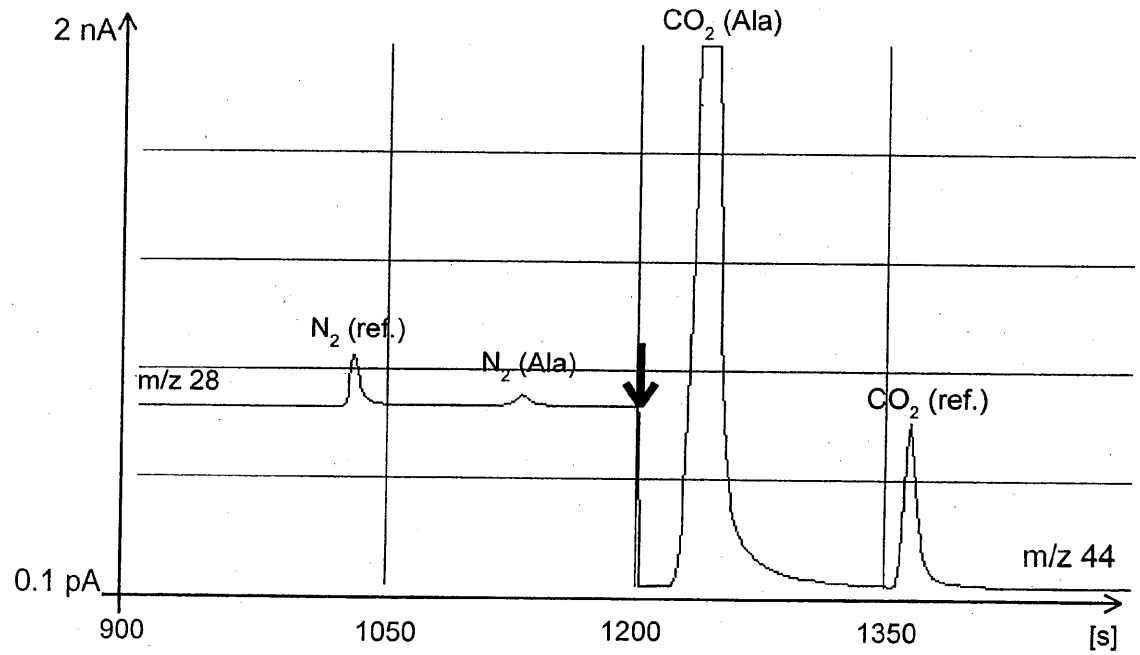

Fig. 6. Dual isotope measurement of $\delta^{15}N$ and $\delta^{13}C$ from alanine (as *N*-acetyl, *O*-propyl derivative) during the same analysis. The arrow indicates when the ion source parameters were switched from nitrogen mode (*m/z* 28) to carbon dioxide mode (*m/z* 44). A 100-s baseline to baseline separation of N_2 from CO_2 was achieved by passing the combustion products past-reduction through a PoraPLOT Q column, held at 35°C. The results obtained from dual-isotope analyses ($\delta^{15}N$, −7.78±0.10‰ vs air; $\delta^{13}C$, −40.22±0.14‰ vs PDB) were in good agreement with those obtained from separate analyses ($\delta^{15}N$, −7.86±0.38‰ vs air; $\delta^{13}C$, −40.20±0.21‰ vs PDB).

GC via a pyrolysis interface including a 5-Å molecular sieve PLOT column to achieve CSIA for ^{2}H of fatty acids. Preliminary $\delta^{2}H$ values for 16:0 and 18:1 fatty acids (as methyl esters) from tuna oil given in a Technical Brochure were -148.5 ± 4.1 and -155.3 ± 1.0‰ (vs. VSMOV), respectively [160].

A different approach to CSIA for H of organic compounds such as ethyl benzene and cyclohexanone was published by Tobias and Brenna. Initially using a two-stage reactor interface (CuO at 850°C followed by Ni held at 950°C) [161] they found that better precision for $\delta^{2}H$ was achieved by employing an empty alumina tube held at about 1150°C [162]. Because their IRMS was not capable of fully resolving analyte $^{2}H^{1}H$ from excess ^{4}He carrier gas, they used a heated Pd filter in conjunction with a make-up pressure unit to prevent He from entering the IRMS while selectively admitting only hydrogen through the Pd foil membrane into the ion source [163].

The measurement of intramolecular variations in isotopic abundance due to kinetic isotope effects during biosynthesis is another recent development to extend the scope of GC–IRMS. The group around Schmidt employed ^{13}C isotope pattern analysis for distinction of natural compounds from corresponding synthetic products [53,164]. In 1997, an on-line pyrolysis system for position-specific isotope analysis (PSIA) of selected compounds from a complex mixture was described in detail by Corso and Brenna [165]. They coupled a GC (GC-1) for sample separation prior to pyrolysis to the GC (GC-2) separating pyrolytic products of the selected sample compound. Furthermore, they installed a valve into GC-2 to permit separated pyrolysis fragments to be admitted to an organic MS for structure analysis of these fragments.

6. Conclusions

Despite recent advances, many fundamental challenges for improved instrumentation still remain, most notably developments leading to (a) quantitative sample conversion to achieve high-precision CSIA for nitrogen; (b) routine CSIA of hydrogen isotopes after gas chromatographic separation; and (c) the routine application of PSIA to detect intramolecular isotope patterns. Continuing improvements in accuracy, precision and abundance sensitivity of GC–IRMS, accompanied by increased user-friendliness, will ensure that this technique will cement its role as an important and unique tool of analytical mass spectrometry.

The sheer number of applications, as well as their wide spectrum clearly demonstrates that state-of-the-art GC–C–IRMS instruments are already powerful tools providing quantitative and qualitative information that cannot be obtained by other means.

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY
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Routine Analysis by High Precision Gas Chromatography/Mass Selective Detector/ Isotope Ratio Mass Spectrometry to 0.1 Parts Per Mil

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Stable isotope methods are potentially quite useful for validating natural or enhanced mineral degradation of contaminants. For this reason, a continuous flow gas chromatograph (GC), isotope ratio mass spectrometer (IRMS) has been coupled with a quadrupole mass selective detector (MSD) to allow simultaneous mass spectral and stable carbon isotope ratio data to be obtained from a single chromatographic analysis. This allows the target contaminant and any extra-cellular degradation intermediates to be both qualified and quantified. Previously acceptable limits of precision (0.3 parts per mil) are undesirable given the small fractionation observed during aerobic degradation. To further understand the fate of organic contaminants and to gain information about the metabolic degradative pathway employed by a microorganism, routine isotopic analyses on a range of analytes have been performed. Quantities of sample producing mass-44 ion beam signal (I^{44}) of 2×10^{-10} to 1×10^{-8} A were analysed. When the IRMS was tuned for high sensitivity, ion source nonlinearities were overcome by peak height correction from an algorithm that was produced using known isotopic standards of varying concentrations. This led to sample accuracy of <0.01‰ and sample precision of 0.1‰. **Copyright © 1999 John Wiley & Sons, Ltd.**

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The ability to elucidate small differences in the stable carbon isotopic composition of organic compounds by means of isotope ratio mass spectrometry[1] provides a powerful tool in studying processes that may discriminate one stable isotope over another. Biological isotope fractionation[2] opens a range of potential applications for this tecnique. This means that isotope effects can be used as a powerful tool for gaining information about chemical reactions and enzyme-catalysed degradation steps.[3–5] A major development in the field was the coupling of a gas chromatograph (GC) to an IRMS[6] by the introduction of a combustion interface (C). The principle difference between a standard IRMS and a GC/IRMS was the continuous on-line nature of the latter. Organic samples of interest can now be isolated and oxidised on-line by passing them through a copper oxide combustion tube. The combustion products are then purified to leave only target CO_2 derived from the sample carbon. This is fed directly into the IRMS, allowing separation and rapid determination of isotopic ratios for a range of organic compounds in complex matrices. However, this innovation initially compromised the precision of the instrument. Instrumental differences from dual inlet mass spectrometers meant that precision of the initial GC/IRMS and later described instruments[7] of 0.5% was lower than the <0.1% reported for the duel inlet instruments.[2] Reasons for the decrease in precision[7,8] included:

(a) the difference in ion beam size between the reference peak and the sample peak due to the capability of the GC/IRMS to analyse a range of analytes from one sample,
(b) the quality and size of the combustion tube packing, and
(c) the performance and design of the cryogenic water trap.

It is therefore obvious that a better understanding of possible instrumental effects will be advantageous to any analytical system[9] especially when it has the complexities of two or more instruments coupled together.

An IRMS has applications in a large area of environmental monitoring.[10,11] However, the complexity of bioremediation systems presents a large variety of possible compounds present in the sub-surface as target contaminants[12] and/or secondary metabolic breakdown products. It would therefore be beneficial to have an analytical system that provides structural information as well as accurate, precise carbon isotopic information about a target analyte from a single sample. Meier-Augenstein *et al.*[13] described an instrument capable of performing this duel analysis by coupling an ion trap detector to a GC/C/IRMS. Our aim was to couple an existing GC/mass selective detector (MSD) system to an IRMS detector thus incorporating a quadropole detector rather than an ion trap detector as described previously.[13] Furthermore, it is desirable to achieve accurate and precise results over a large range of sample sizes.

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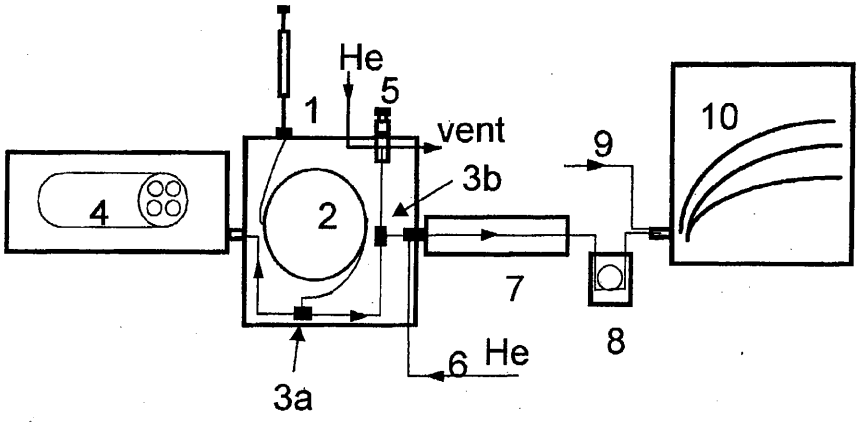

Figure 1. Instrument setup: 1 Injection port, 2 Gas chromatographic column, 3 a, b Split valves, 4 MSD with quadrupole filter, 5 Heart split valve, 6 Sample line helium, 7 combustion tube, 8 water trap, 9 Reference gas, 10 IRMS.

Metabolic intermediate products from degradation pathways can be formed in small amounts[14] or be present for a short period of time.[15,16] Sample considerations, coupled with the problem that some of the original chromatographic sample is diverted to the MSD, necessitates analysis of small samples producing mass 44 ion beam signals (I^{44}) between 1×10^{-10} and 1×10^{-8} A. At the time of writing the manufacturer's recommendation is that, after optimisation, analysis is only non-signal dependent over one order of magnitude (1×10^{-9} to 1×10^{-8} A).

In this paper we describe a GC/IRMS system coupled with a mass selective detector (MSD) that provides routine quantitative and qualitative analysis from the MSD and rapid carbon isotopic analysis using the IRMS (Fig. 1). We also outline a series of analyses on a variety of compounds and the development of a simple correction to allow routine analysis of trace compounds ($I^{44} = 2 \times 10^{-10}$–$1 \times 10^{-8}$ A) to an accuracy of 0.01‰ and to a precision of 0.1‰.

EXPERIMENTAL

Instrumentation

Figure 1 outlines the GC/MSD/IRMS system currently operating in the Environmental Research Centre (EERC) at Queen's University Belfast. The chromatographic unit is a CE 8000 Top gas chromatograph fitted with a Cryo 820 sub-ambient oven temperature unit (Thermoquest, Milan, Italy). The column effluent is then split inside the oven by a SGE GVF16-(2)004 ferrule (Fig. 1, item 3a). One output from this ferrule flows into the MD800 mass selective detector (Fig. 1, item 4) via a measured length of fused capillary tubing. The second effluent flow is connected to a second SGE GCF16(2)004 ferrule (Fig. 1, item 3b). This then splits the flow into two further capillaries. One of the capillaries flows into the heart split valve (Fig. 1, item 5) and the other into the combustion interface (Fig. 1, item 7). The sample is oxidised by fine CuO granules (0.61 mm) and passed through a cryogenic water trap (Fig. 1, item 8). This removes water from the oxidation products allowing purified CO_2 to flow into a bench-top VG Isoprime® IRMS (Micromass UK Ltd., Manchester, UK) (Fig. 1, item 10).

Standard preparation

Four compounds, decane, undecane, dodecane and methyl decanoate, (VG mix, obtained from Micromass UK Ltd.) were dissolved in hexane (15 ng/mL). This is used as an internal laboratory standard and routinely analysed as a quality assurance/control (QA/QC) to check the system.

Isotopically known Carrara marble[17] derived CO_2 standards were made by dissolving a powdered aliquot of Carrara marble in excess phosphoric acid (95%). The acidification for the liberation of CO_2 was carried out in pre-evacuated, gas-tight vials. The vials were then kept at 25 °C for at least 4 hours to ensure CO_2 evolution under equilibrium conditions.

The phenol standards were obtained by dissolution of pure compound in ethyl acetate (0.47 mg/mL). The $\delta^{13}C$ value of phenol was also measured on a Prism III dual inlet IRMS. Ethyl acetate was used as the solvent to compare with the phenol extraction method used to study phenol utilisation and isotopic fractionation.[18]

Chromatographic conditions

The VG mix and phenol samples were injected with varying split ratios (30:1–166:1) and sample sizes (1–10μL) to give the desired I^{44} intensity. Investigations have shown that split injection causes no isotopic fractionation.[9] The CO_2 samples were injected in split mode using a gas-tight syringe. Helium (3 mL/min) was used as the carrier gas. The GC columns used were a DB5® 15 m × 0.22 mm (J & W Scientific, Folsom, CA, USA) for organic samples and a CP-Molsieve 5 Å 25 m × 0.32 mm (Chrompack, Middelburg, The Netherlands) for CO_2 samples.

RESULTS

Instrumental conditions

The identification of an analyte by its mass spectrum and the carbon isotopic ratio obtained from the same compound are unambiguously linked, because both sets of information are obtained from one injection. Figure 2 suggests that the column effluent is split effectively between the two detectors. The nonlinear trend between MSD and IRMS peak areas is thought to be an artefact of the MSD response

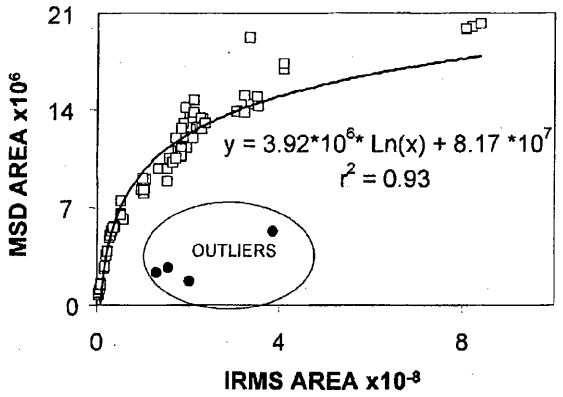

Figure 2. Trend of the peak areas of the IRMS and MSD.

Table 1. Measured $\delta^{13}C$ values of analytes at constant ^{44}I beam (2μL injection, split 20:1) in comparison to their theoretical values including retention times (R_t) on the MSD and IRMS

Component	Decane	Undecane	Dodecane	Methyl decanoate
	−28.34	−26.61	−28.50	−30.42
	−28.56	−26.78	−28.65	−30.72
	−28.54	−26.71	−28.57	−30.50
	−28.43	−26.56	−28.56	−30.56
	−28.57	−26.72	−28.59	−30.51
Average	−28.49	−26.68	−28.57	−30.54
1σ	0.10	0.09	0.05	0.11
Theoretical	−28.61	−26.70	−28.63	−30.47
Difference	0.12	0.02	0.06	−0.07
MSD R_t	300.6	387.6	469.8	564.6
1σ†	1.2	0.6	0.6	0.6
IRMS R_t	333.5	420	501.6	596.6
1σ	2.1	2.5	2.8	2.7

† 1σ - one standard deviation.

to increasing sample size. The MSD response is not linear above 1.4×10^7 total ion count (TIC) and this therefore creates the nonlinear effect at higher sample concentrations seen in Fig. 2.

Although the column flow is split and forced to flow through an excess length of capillary (40 cm × 75 μm), the chromatographic resolution is not degraded. Likewise, despite being split twice by union valves, the resolution of the spectrum from the IRMS is not impaired, nor the integrity of the isotopic data harmed (Table 1). The main difficulty in using an MSD as a second detector with a GC-IRMS is obtaining the correct pressure balance.

With a normal GC-IRMS system, after solvent peak elution, the heart split valve closes, the sample line helium builds up a back pressure and forces the column effluent to flow with this excess helium along the combustion interface to the IRMS detector. However, we have added an extra capillary from the MSD that exerts a negative pressure on the initial split at the end of the column. This subsequently creates a delicate balance between three exerting pressures: (1) the negative pressure created by the MSD, (2) the pressure flow of column effluent, and (3) the pressure flow of sample line helium.

The balance of these pressures can be calculated using Eqn. 1 or empirically. A major problem to be noted was the difficulty in placing the two capillaries in the union on the opposite side of the column fitting to achieve a balanced flow to both the MSD and the second split. Once this was achieved, the ideal column flow was elucidated (2.0–3.5mL/min) to maintain chromatographic integrity and speed of analysis. The length of capillary from the first split to the MSD interface had to be of the correct diameter to maintain laminar flow. The relationship is outlined in the following modified Poiseuille's equation:

$$V_0 = 50747 \cdot \frac{d^4}{\eta l} \cdot \frac{\{(p_i + 14.7)^2 - (p_o + 14.7)^2\}}{(p_o + 14.7)} \cdot \frac{p_o}{p_m} \cdot \frac{(t_m + 273)}{(t_c + 273)} \quad (1)$$

where V_0 is the carrier gas volume (mL/min), η is the dynamic viscosity of the carrier gas (micropoises), l is the length of the capillary column (m), p_i is the carrier gas inlet pressure above atmospheric (psi), p_o is the carrier gas outlet pressure (psi), p_m is the pressure at which the measurement is made, t_m is the temperature at which the measurement can be made (°C) and t_c is the temperature in the column. The important factor to note from Eqn. 1 is that the relationship between flow through the capillary and the diameter is proportional to d^4. This means that for a small decrease in the diameter of the capillary to the MSD we observe a substantial decrease in the effective vacuum exerted on the split union.

Organic samples

Initially the VG mix was used to calibrate the instrument. The IRMS I^{44} sensitivity is greatly variable depending on variations in the set of source tuning parameters. This, coupled with the fact that the flow is split at the injection port, first union, and at the open split at the end of the combustion tube, suggests that the absolute mass of carbon entering the IRMS source is difficult to quantify. The sample is therefore described as nmoles on-column. A common term encountered in the literature describing the performance of an IRMS instrument is the 'linearity'.[19] It is the authors' opinion that this term is vague and incorrectly used. The term 'linear' should describe the fact that the ion beam intensity is related by a linear (1st order polynomial) variation to the $\delta^{13}C$ value produced by that signal. IRMS manufacturers and operators generally use the term 'linear' to mean that the measured $\delta^{13}C$ value produced by the IRMS is independent of I^{44} intensity. A more correct term for this is signal independence and this is used herein. Table 1 shows that a 2μL injection at a split of 20:1 of VG mix (1.5 ng on-column) is required for an I^{44} of 6.5×10^{-9} A. This is well within the manufacturer's specified signal independent range. The carbon isotopic ratios of the four compounds showed an average precision of 0.09‰ (1σ) and an accuracy error of <0.03‰. Table 1 also shows that the retention times on both detectors were very reproducible.

As stated earlier, our analytical system consists of a GC coupled to two detectors. It is therefore of prime importance to optimise the sensitivity of the IRMS to counter the effect of the extra split of column effluent to the MSD (Fig. 1, item 3a). This was achieved by setting the source tuning parameters to values that assured a high sensitivity response. The tuning parameters manipulated were the extraction voltage, half-plate differential voltage, z focus, ion repeller and electron volt setting.

Results outlined below demonstrate that the instrument